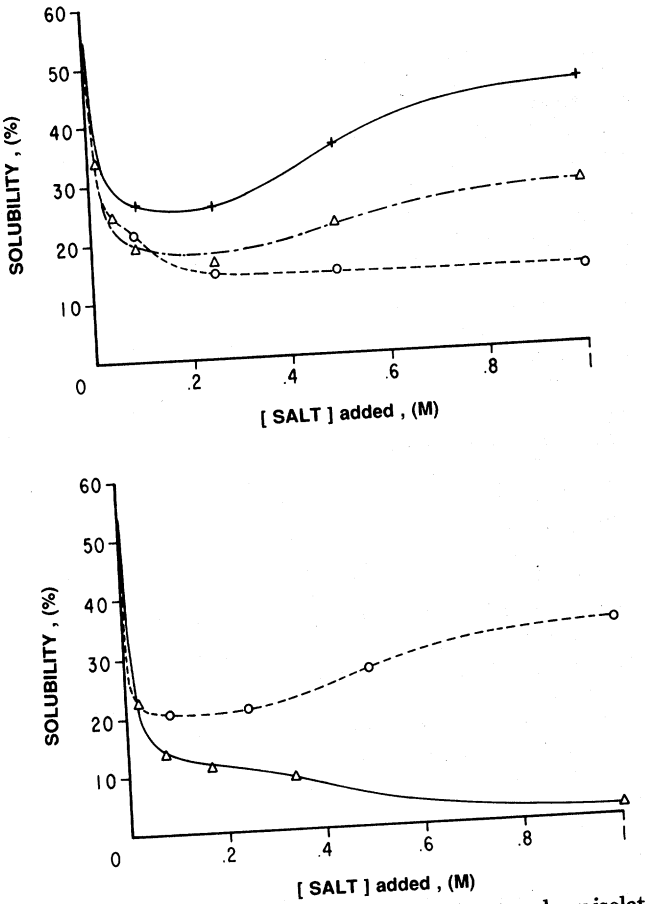

Figure 2. Salt-induced solubility profile of denatured soy isolate: a, same as in Figure 1a; b, same as in Figure 1b.

however should not be interpreted literally as only a simple binding site, since it is well-known that multiple binding sites with exactly the same equilibrium constant yield only a single binding isotherm (Tanford, 1961). Hence, a value of n or m represents a class of protein binding sites rather than a single binding site linked to the solubility change of the protein.

The salting-out solubility, S_1, shows no trend with respect to the type of anion used whereas a slight trend may exist for the salting-in solubility, S_2, which will be discussed later in this paper. Since soy isolates at neutral pH are thought to have a net negative charge (Shen, 1981), the above results can easily be interpreted in terms of an isoelectric binding model; i.e., salt cations bind to negative sites on a protein surface with an average constant of κ_1 and produce a species of zero net charge with a corresponding solubility of S_1. The further salting-in of the protein can be thought of as either only cations binding, κ_2, to the unbound negative protein sites yielding a species, S_2, with a net positive charge or both salt cations and anions binding, κ_2, to corresponding protein negatively and positively charged sites, yielding a species with a zero or negative charge. The values of S_1 and S_2 are higher for NaI, 43 ± 2 and 74 ± 4, respectively, than the chloride, bromide, and nitrate values, approximately 50 and 4 L/mol, respectively.

The solubilities of native soy isolate, S_1, are high in $(NH_4)_2SO_4$ and Na_2SO_4 in comparison with the other salts mentioned in Table I. In addition, the κ_1 value is significantly higher but the κ_2 value is much smaller than the rest. However, the n and m values, 4 and 4, respectively, were different from the corresponding 1 and 2 values for the other salts. Hence, the sulfate salt-induced solubility profile for native soy isolate will only be presented and not compared with the others in this series. Nevertheless, it should be noted that even though the shape of the sulfate profile (Figure 1b) is different from the rest of the salts (Figure 1a,b), inasmuch as the sulfate solubility decreases to a plateau at approximately 0.1 M and then further decreases to a limiting value (rather than decreasing to a minimum followed by an increase to a limiting value), this mechanism, quantitated by eq 4, can easily describe both solubility profiles. The only difference between these two profiles is that $S_2 < S_1$ for sulfate and $S_2 > S_1$ for the other salts.

Table III. Soy Isolate Solubility (S_1 and S_2) and Molar Surface Tension Increment (σ)

salt	$\sigma \times 10^3$ [a]	S_{1N}, [b] %	S_{2N}, [c] %	S_{1D}, [d] %	S_{2D}, [e] %
NH_4NO_3	0.85	29 ± 12	63 ± 13	20.2 ± 0.1	34.7 ± 0.1
NaI	1.02	43 ± 2	74 ± 4	23.4 ± 0.1	46.1 ± 0.2
NH_4Br	1.14	28 ± 10	62 ± 9	16 ± 1	28 ± 4
NH_4Cl	1.39	31 ± 2	59 ± 4	21 ± 4	13 ± 8
NaCl	1.64	31 ± 2	59 ± 9	21 ± 4	13 ± 8
$(NH_4)_2SO_4$	2.16	55.1 ± 0.3	44 ± 1	10 ± 1	1.0 ± 0.5
Na_2SO_4	2.73	55.1 ± 0.3	44 ± 1	10 ± 1	1.0 ± 0.5

[a] in dyn·g/cm·mol; values from Melander and Horvath (1977). [b] S_{1N} = salting-out for native protein. [c] S_{2N} = salting-in for native protein. [d] S_{1D} = salting-out for denatured protein. [e] S_{2D} = salting-in for denatured protein.

Table II shows the binding and solubility parameters for the heat-denatured soy isolate derived from the data in Figure 2a,b. With the exception of nitrate, all κ_1 values are invariant with the type of salt used in this study. All κ_2 values with the exception of chloride show a similar invariant behavior. The latter difference is most likely due to experimental error, and the conclusion that the salt binding is invariant to the lyotropic series is warranted especially since all n and m values were found to be equal to 1 and 4, respectively. Inspection of the κ_1 values from Tables I and II for native and denatured soy isolates shows significantly higher values for the latter form of the isolate. This effect is consistent with the notion that a protein in its native state has a more compact tertiary structure than the corresponding denatured form (Tanford, 1961). Hence, it is reasonable to conclude that κ_1 and S_1 reflect an isoelectric salt-binding mechanism. This mechanism is also corroborated by the fact that S_1 values from the native protein (Table I) are somewhat larger in magnitude than those from the denatured form (Table II), while remaining relatively invariant to the type of salt used in this study with no trend for S_1 in either Tables I or II existing with the lyotropic series.

S_2 for the denatured soy isolate (Table II), however, strongly follows the Höffmeister series for proteins. The solubility increases dramatically in the order $SO_4 < Cl < Br < NO_3 < I$. An attempt was made to quantitate this lyotropic trend of S_2 with either hydrophobic forces or anion binding. Although Melander and Horvath (1977a,b) postulated that the surface tension increment of the salt and the hydrophobic surface area are responsible for the salting-out phenomenon, we have compared all S_1 and S_2 values for both native and denatured soy isolate in Table III along with the molar surface tension increment, σ (column 2, Table III) (Melander and Horvath, 1977a,b), in increasing order of magnitude. No trend can be observed for any S_1 and S_2 value with increasing σ. Hence, it appears that the surface tension and possibly hydrophobic interactions have little or no effect on the salt-induced solubility change of soy isolates. This is surprising since there exists a strong trend in S_2 for denatured soy with varying types of added salts, and it has been well

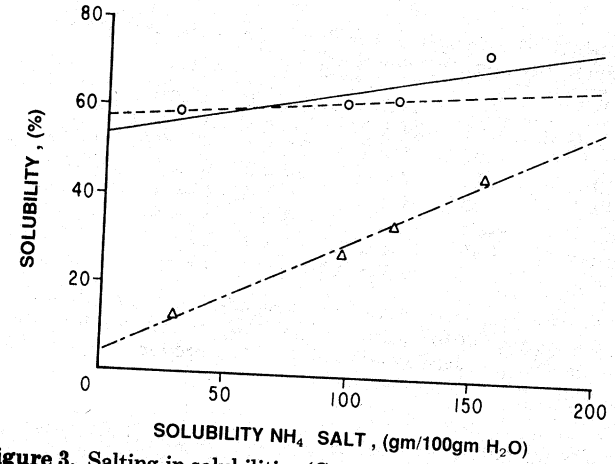

Figure 3. Salting-in solubilities (S_2 from Tables I and II) versus literature solubilities of ammonium salts with corresponding anions (Wagman, 1968) of Cl^-, Br^-, NO_3^-, and I^- (left to right). Key: O, native soy isolate; —, least squares straight line for first three points only; ---, least squares straight line for all four points; Δ, denatured soy isolate; –·–, least-squares straight line for denatured soy isolate data.

documented that hydrophobic groups are more exposed to the solvent when protein denaturation has occurred (Tanford, 1961).

To test the concept of anion binding being the predominate contribution to S_2 for the denatured protein, S_2 values were plotted against literature values for the solubility of ammonium chloride, bromide, nitrate, and iodide (Wagman, 1968) and are presented in Figure 3 as the open triangles. The dot-dashed line in Figure 3 is the least-squares straight line, which is in excellent agreement with the experimental values with a root-mean-square deviation (rms) of 1.2, yielding a slope of 0.26 ± 0.02 and an intercept close to zero, namely, 4 ± 2. This strong linear dependency of S_2 with the solubilities of corresponding ammonium salt solubility is a strong indication that for the heat-denatured soy isolate the dominant contribution to the salting-in phenomenon is the salt anion binding to the positively charged amine groups on the protein surface.

A smaller but somewhat significant trend for S_2 versus the ammonium salts solubilites was also observed for native soy isolate (open circles in Figure 3). Linear regression analyses on all four S_2 values yield an rms value of 3.5 with a slope of 0.11 ± 0.05 and a large intercept of 54 ± 5. Better statistics are obtainable if the iodide point is eliminated, which yielded a rms of 0.3 with a slope of 0.045 ± 0.003 and an intercept of 57.6 ± 0.1. However, at this time there is no valid reason for the incorporation or deletion of the iodide value. Furthermore, since both intercept values for the native soy are much larger than the denatured soy, i.e. approximately 50% verses 4%, respectively, it can be concluded that anion binding has a small but real effect on the salting-in of native soy isolate. A possible explanation is that only a limited amount of anion binding can occur in native soy because of its closed tertiary structure. This hypothesis is consistent with the value of m for native protein, 2, being lower than the denatured form, 4. If anion binding does not occur during the salting-in process and only salt cations are bound to native soy, then S_2 species would have a net positive charge assuming that the S_1 state has a net charge of zero. Hence, a positive net charge of the S_2 species would indeed have a relatively large solubility, resulting in an S_2 value that is invariant with the Höffmeister series and large in magnitude. Therefore, for native soy isolate the predominate driving force for the salting-in process appears to be caused only by the cation binding, leading to a protein complex with an apparent positive charge. For the heat-denatured soy isolate, the salting-in process is most likely caused by both salt anion and cation binding to the corresponding protein positive and negative sites, respectively yielding a complex with a net zero charge. The salting-out process of both forms of the soy isolate is caused by salt cation binding to a protein with a net negative charge leading to isoelectric precipitation.

In conclusion, the above results are a strong indication that the binding of salt cations and/or anions to proteins has a major influence on protein solubility, even though the magnitude of the binding constants is small. Furthermore, the binding parameters can be influenced by the secondary and tertiary structures of the protein, as in the present case for native and heat-denatured soy isolates. However, more studies of this type should be performed on other protein systems and with a variety of salts to test the general validity of this approach. Such studies are now in progress at this laboratory.

Registry No. NaCl, 7647-14-5; NH_4Cl, 12125-02-9; NH_4Br, 12124-97-9; NH_4NO_3, 6484-52-2; NaI, 7681-82-5; $(NH_4)_2SO_4$, 7783-20-2; Na_2SO_4, 7757-82-6.

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Use of Thermodynamic Linkage To Study the Salt-Induced Solubility Change of Soybean Isolate

Thomas F. Kumosinski

Salt-induced solubility profiles of native and heat-denatured soy isolate reportedly were uninterpretable by the theory of Melander and Horvath. An explanation had been sought by a qualitative extension of the latter theory, discounting the notion that salt binding to various charged protein sites causes salting-out and subsequent salting-in. Reevaluation of the data, using nonlinear regression analysis with Wyman's theory of thermodynamic linkage, demonstrated that protein solubilities can now be linked to the free energy of salt binding. All profiles could then be described quantitatively by binding constants and solubilities: k_1 and S_1, respectively, for salting-out; k_2 and S_2 for salting-in. k_1 always exceeded k_2, but k_1, k_2, and S_1 followed no apparent trend with type of salt. However, k_1 was significantly larger for denatured than for native soy; S_2 correlated with the lyotropic series, strongly for the denatured, less so for the native isolate.

A report by Shen (1981) has shown that the salt-dependent variation in the solubility of native and denatured soy bean isolates cannot be explained by the theory of Melander and Horvath (1977a,b). This latter theory predicts that at low salt concentration the solubility of a protein should increase because of an electrostatic contribution to the free energy. At higher salt concentrations a salting-out free energy predominates because of the increase of surface tension of the salt solution and the exposed hydrophobic surface area of the protein. However, Shen (1981) found that for both native and heat-denatured soybean isolates the opposite phenomenon occurs; i.e., the protein solubility decreases with added salt to a minimum value and then increases to a constant value at a salt concentration of approximately one molar. In addition, he found the shape of this salt-induced solubility profile is the same for both the native and denatured forms of the isolate when sodium or ammonium chloride, ammonium bromide, ammonium nitrate, and sodium iodide is used. Moreover, he found the limiting values of solubility at high concentrations to follow the usual lyotropic or Höfmeister series. Shen suggested that these results could be explained by an increase in the hydrophobic surface area of the protein due to protein self-association with added salt, followed by a salting-in term caused by an increase in the dipole moment of the protein due to a nonspecific solvation effect at higher salt concentrations. He discounted the possibility that ion binding causes the net charge of the protein to be zero, resulting in salting-out; further salt binding causes salting-in. The rationale for this decision was that the binding of either NH_4^+ or Na^+ to soy proteins is negligible. Other investigators (Steinhardt and Reynolds, 1979) have shown sodium and ammonium salts to significantly bind to proteins. Therefore, using the concept of Ockham's razor, this ion-binding model appears to be more plausible than the more cumbersome, qualitative description offered by Shen (1981), and it should be tested quantitatively before discounting it. For these reasons we have reevaluated the results of Shen using equations adapted from Wyman's theory of linked functions (Wyman, 1964).

Here, the solubility of a protein was thermodynamically linked to its salt-binding capacity. Shen's solubility profiles were fitted to these derived equations by nonlinear regression analysis. The stoichiometry with corresponding binding constant as well as salting-out and salting-in parameters were obtained.

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METHODS

Solubility Data. Three-time enlargements of published solubility figures 8 and 9 of Shen (1981) were digitized with use of a coordinate digitizer interfaced to a Modcomp Classic computer, to maintain a high degree of precision since the exact numerical values of solubility were not reported.

Shen (1981) reported that the native soy protein isolate (NPI) was prepared with 1 part commercial defatted soy flakes to 10 parts NaOH (pH 9–10). The extract was then adjusted to pH 4.5 with dilute HCl to precipitate the curd. The curd was washed three times with water, at pH 7.0, and freeze-dried. He then reported this fraction to be essentially native protein as validated by intrinsic viscosity, optical rotation, and differential scanning calorimetry (DSC) measurements.

The denatured protein isolate (DPI) was prepared by resuspending a portion of the pH 7.0 curd, heating at temperatures above 90 °C, and spray drying. Shen also validated this form to be denatured by DSC measurements.

He modified his solubility measurements (Shen, 1976) by increasing the centrifugation time from 20 to 40 min in order to remove all particles with a $S_{20,w} > 70$ from the solutions.

Theory and Data Analysis. The observed biphasic nature of the salt-induced solubility profiles of soy isolates (Figure 1a,b) can be described mathematically if we assume that there are essentially two classes of binding sites for ligands responsible for the sequential salting-out and salting-in processes. Therefore, the concept of Wyman's linked functions (1964) can be used to treat these processes according to eq 1, where P is the unbound protein, X is

$$\underset{(S_0)}{\mathrm{P}} + n\mathrm{X} \overset{\kappa_1^n}{\rightleftharpoons} \underset{(S_1)}{\mathrm{PX}_n} + m\mathrm{X} \overset{\kappa_2^m}{\rightleftharpoons} \underset{(S_2)}{\mathrm{PX}_n\mathrm{X}_m} \qquad (1)$$

the free salt, n and m are the number of X moles bound to species PX_n and PX_nX_m, and S_0, S_1, and S_2 are the solubilities of the species indicated. For this study S_1 and S_2 will be relative to S_0. The mathematical relationship representing the above stoichiometry can be represented according to eq 2, where S_{app} is the apparent protein

$$S_{app} = S_0 f(\mathrm{P}) + S_1 f(\mathrm{PX}_n) + S_2 f(\mathrm{PX}_n\mathrm{X}_m) \qquad (2)$$

solubility at a given salt concentration (X_T), $f(i)$ are the protein fractional components of species i, and the S values

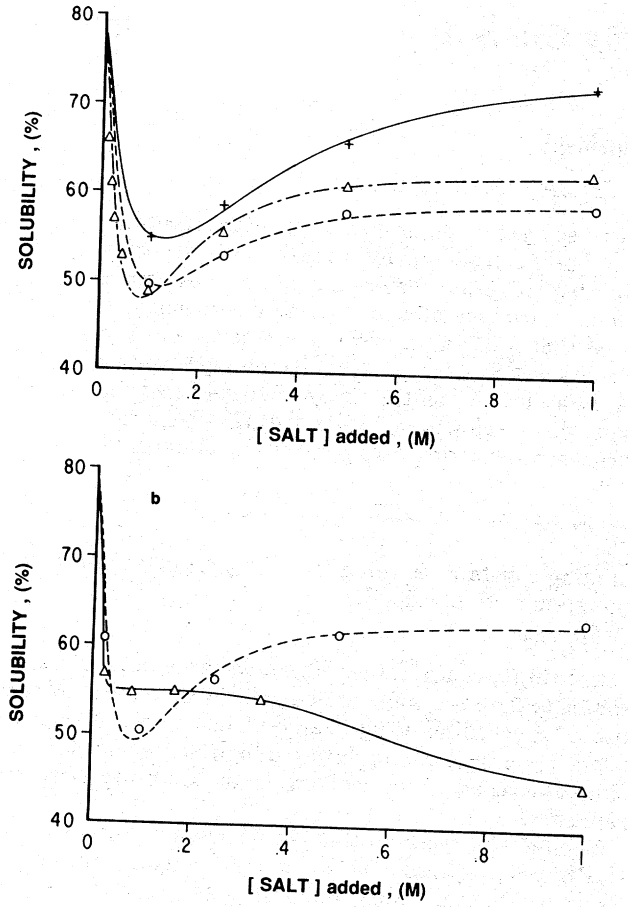

Figure 1. Salt-induced solubility profile of native soy isolate. (a) Key: O, solubility data for NaCl or NH_4; ---, theoretical curve for NaCl or NH_4 data; Δ, solubility data for NH_4Br; -·-, theoretical curve for NH_4Br data; +, solubility data for NaI; —, theoretical curve for NaI data. (b) Key: O, solubility data for NH_4NO_3; ---, theoretical curve for NH_4NO_3 data; Δ, solubility data in $(NH_4)_2SO_4$ or Na_2SO_4; —, theoretical curve for $(NH_4)_2SO_4$ or Na_2SO_4 data.

are as previously defined. Incorporation of the salt binding equilibrium constants as defined by (1) and (2) easily yields eq 3, where p is the concentration in percent of the un-

$$S_{app} = \frac{S_0 p}{p + \kappa_1{}^n p X^n} + \frac{S_1 \kappa_1{}^n p X^n}{p + \kappa_1{}^n p X^n} + \frac{(S_2 - S_1)\kappa_2{}^m p X^m}{p + \kappa_2{}^m p X^m} \quad (3)$$

bound protein and X is the concentration of unbound salt. Cancellation of common terms yields eq 4. It should be

$$S_{app} = \frac{S_0}{1 + \kappa_1{}^n X^n} + \frac{S_1 \kappa_1{}^n X^n}{1 + \kappa_1{}^n X^n} + \frac{(S_2 - S_1)\kappa_2{}^m X^m}{1 + \kappa_2{}^m X^m} \quad (4)$$

stressed here that the above expression is valid for sequential binding, i.e. $\kappa_1 > \kappa_2$, and n sites saturate prior to the binding of m sites on the protein. Also, for n or m greater than 1, note that κ_1 and κ_2 represent an average value for each of the n or m binding sites. In reality, n or m moles of salt will bind with only one equilibrium constant (K_1): i.e. $K_1 = \kappa_1{}^n$ and $K_2 = \kappa_2{}^m$.

Now, since the total salt concentration, X_T, is the sum of the free salt concentration, X, and the concentration of the bound salt of both species PX_n and PX_nX_m, it can be shown that

$$X_T = X\left[1 + \frac{n\kappa_1{}^n P_T X^{n-1}}{1 + \kappa_1{}^n X^n} + \frac{m\kappa_2{}^m P_T X^{m-1}}{1 + \kappa_2{}^m X^m}\right] \quad (5)$$

Table I. Native Soy Protein Isolate

salt	κ_2, L/mol	κ_2, L/mol	S_1, %	S_2, %	n	m
NaCl, NH_4Cl	41 ± 9	4.7 ± 0.4	31 ± 2	59 ± 4	1	2
NH_4Br	53 ± 16	6 ± 2	28 ± 10	62 ± 09	1	2
NH_4NO_3	51 ± 17	6 ± 2	29 ± 12	63 ± 13	1	2
NaI	50 ± 6	2.9 ± 0.3	43 ± 2	74 ± 4	1	2
Na_2SO_4, $(NH_4)_2SO_4$	100 ± 1	1.6 ± 0.1	55.1 ± 0.3	44 ± 1	4	4

Table II. Denatured Soy Protein Isolate

salt	κ_2, L/mol	κ_2, L/mol	S_1, %	S_2, %	n	m
NaCl, NH_4Cl	383 ± 43	8 ± 4	21 ± 4	13 ± 8	1	4
NH_4Br	240 ± 22	1.9 ± 0.4	16 ± 1	28 ± 4	1	4
NH_4NO_3	265 ± 5	2.0 ± 0.1	20.2 ± 0.1	34.7 ± 0.1	1	4
NaI	238 ± 1	2.0 ± 0.1	23.4 ± 0.1	46.1 ± 0.2	1	4
Na_2SO_4, $(NH_4)_2SO_4$	273 ± 18	2.2 ± 0.6	10 ± 1	1.0 ± 0.5	1	4

where P_T is the total concentration of protein. From eq 5 it can be seen easily that $X_T = X$ when P_T is small relative to X. For these experiments this assumption is reasonable because of the concentration range of the total salt and the large molecular weight of the soy protein isolate. Therefore, since the total salt concentration could be used in (4) instead of the free concentration, the salt-induced solubility profiles can be directly analyzed with a Gauss–Newton nonlinear regression analysis program developed at this laboratory. All solubility profiles were analyzed by fixing the values of n and m and calculating the best least-squares fit for the optimum evaluated κ_1 and κ_2 values. The n and m values were then fixed to new values, and the whole procedure was repeated. The n and m values, which yielded the minimum root-mean-square value for the analysis, were then reported.

RESULTS AND DISCUSSION

The salt-induced solubility data of Shen were analyzed according to eq 4 using nonlinear regression analysis for the native soy isolate at neutral pH. The results are shown in Figure 1a for NaCl or NH_4Cl, NH_4Br, and NaI, and Figure 1b for NH_4NO_3 and $(NH_4)_2SO_4$ or Na_2SO_4. Figure 2a,b shows the best fits for denatured soy isolate at neutral pH with the same corresponding salts as in Figure 1a,b. As it can easily be seen, the resulting theoretical curves for the profiles in Figures 1 and 2 are in excellent agreement with all the solubility data. The curves, which represent the nonlinear regression best fit for the lowest root-mean-square values for fixed values of n and m, are all within 1 relative standard deviation of 2% from the experimental data. The final values with corresponding standard errors of the salting-out binding constant, κ_1, the salting-in binding constant, κ_2, the salting-out solubility, S_1, the salting-in solubility, S_2, and the values of n and m, i.e. the number of moles of salt bound to S_1 species for n and to S_2 species for m, are presented in Table I for the native soy isolate and Table II for the denatured soy isolate.

Once again, it must be stressed that the κ values are not the actual stoichiometry equilibrium binding constants. They are merely average values representative of only 1 mol of salt bound to one protein site. The actual equilibrium constants are calculated by raising the κ value to the corresponding n or m exponent. As seen in Table I, the salting-out constant, κ_1, is essentially invariant within experimental error for NaCl, NH_4Cl, NH_4Br, and NH_4NO_3 as well as NaI. Also, little change in values for κ_2, the salting-in equilibrium constant, is observable for the same lyotropic series of salts. The n and m values are also the same; i.e., $n = 1$ and $m = 2$ for all of the above salts. The relatively low values of 1 and 2 for n and m, respectively,